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OBSERVATIONS ON THREE SPECIES OF JELLYFISHES FROM CHESAPEAKE BAY WITH SPECIAL REFERENCE TO THEIR TOXINS. I. *CHRYSAORA* (DACTYLOMETRA) *QUINQUECIRRHA* [1]

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Chrysaora (Dactylometra) quinquecirrha, the stinging nettle, has attracted the attention of vacationers in the Chesapeake Bay and other areas for many years because of the irritating and sometimes serious nature of its stings. At the time the present work was initiated there was no published information on the nature of the toxic agent of this species. Various terms had been applied earlier to the toxins of different Cnidaria but it is not certain that the extracted substances, *e.g.*, hypnotoxin, congestin, thallasin, *etc.* were isolated from the nematocyst capsules or that they were pure substances. Halstead (1965) has reviewed most of the important literature on the poisonous Cnidaria up to 1965.

Lane and Dodge (1958) and Lane (1960) isolated the contents of the nematocyst capsules of *Physalia physalis* (Portuguese man-of-war) and determined that these appeared to be protein in nature. Welsh (1961), page 180, states, "Much evidence indicates that the paralyzing edema-producing action of coelenterate toxins is due in large measure to a protein component(s)." Subsequently other investigators (Burnett, Stone, Pierce, Cargo, Layne and Sutton, 1968; Shapiro, 1968; Endean, Duchemin, McColm and Fraser, 1968; Crone and Keen, 1969) reach the same conclusion.

Burnett *et al.* (1968) isolated four types of nematocysts from *C. quinquecirrha* and state, page 336, "Almost all the toxic activity was localized in sediments of chemically or physically ruptured nematocyst suspensions," and conclude "that the toxin is membrane bound." However, Barnes (1967) collected the toxins of *Chironex fleckeri* and *Chiropsalmus quadrigatus* after discharge of nematocysts through a membrane (isolated human amnion) and found them to be a fluid. Endean *et al.* (1968) obtained evidence that the toxin of *C. fleckeri* is intracapsular and indicate that the capsules themselves are non-toxic.

Many of the observations reported in the present paper have been made over the past six years but the work on toxins was begun in July, 1967. This project involved (1) the extraction and isolation of the nematocyst toxins of the stinging nettle (*Chrysaora quinquecirrha*), the clover leaf jelly (*Aurelia aurita*), and the pink or lion's mane jellyfish (*Cyanea capillata*), all of which are common in the Chesapeake Bay and parts of its tributaries at certain seasons of the year; (2) toxicity experiments; and (3) the determination of the chemical nature of the toxins.

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This paper reports on the observations and experiments on the summer nettle (*C. quinquecirrha*). Research in progress with *A. aurita* and *C. capillata* will be published later.

Materials and Methods

Nettles were collected during the summer months in the Rappahannock River and Chesapeake Bay (salinity approximately 1.5%) near Deltaville, Virginia. Only the tentacles and oral lobes were used. Usually six to eight gallons of this material were processed at one time.

The following procedure for the extraction of toxin is a modification of that used by Lane and Dodge (1958) for *Physalia*. The *Chrysaora* material was allowed to autolyze in the refrigerator at 5° C for 24 to 48 hours after which it was diluted with an equal volume of 1.5% NaCl solution. This was strained through Marquisette Nylon Netting (20 openings/inch) and then through Swiss silk bolting cloth (No. 12, 125 mesh). The screened suspension was permitted to settle at 5° C in the refrigerator for 24 hours, the supernatant decanted, and discarded. The residue, consisting of nematocysts and cellular debris, was centrifuged at 7000 rpm (6000 *g*) for 15 minutes. The supernatant was discarded and an equal volume of 1.5% NaCl solution was added to the residue. The centrifuge tubes were gently shaken to loosen the cellular debris overlaying the nematocysts which firmly adhered to the bottoms of the tubes. This process was repeated several times. Each time the loosened debris was poured off. The residue was then thoroughly mixed with 1.5% NaCl solution, recentrifuged at 7000 rpm for 15 minutes, the supernatant decanted, and the above described process of washing repeated until a mass consisting almost entirely of undischarged nematocysts was secured. The whole process was continued until the supernatant was Biuret negative.

Initially 5 ml of nematocysts was mixed with 35 ml of 1.5% NaCl solution and homogenized in a Potter-Elvejhem homogenizer to disrupt the nematocysts and release their contents. However, since this was laborious and time consuming, several other methods of rupturing the capsules were tried: osmotic pressure change through the use of distilled water and ultrasonic rupture. The procedure finally adopted was that of repeated homogenization of a frozen suspension of nematocysts in a Potter-Elvejhem homogenizer. In this way 75% or more of the nematocyst capsules were ruptured or discharged, liberating the contents.

The homogenate was centrifuged in early experiments at 9000 rpm (10,000 *g*), in later ones at 18,500 rpm (40,000 *g*), for 1 hour. Most of the strongly Biuret positive supernatant was carefully pipetted off and frozen until used in toxicity and electrophoresis experiments. The residue, consisting of undischarged nematocysts, large amounts of capsular debris, and tubes was repeatedly washed in 1.5% NaCl solution and repeatedly centrifuged until the wash solution was Biuret negative. This residue was frozen for later use.

Some of the supernatant after centrifugation at 9000 rpm was pipetted off and recentrifuged at 18,500 rpm for 1 hour. The small amount of residue, consisting of microscopically fine particulate matter, was used in one of the toxicity experiments. The supernatant was treated with ammonium sulfate, centrifuged at 7000 rpm for 15 minutes, and the resulting pellet was dialyzed in standard dialysis tubing. The dialysate was used in one of the experiments.

In all toxicity experiments materials for injection were made up in 0.9% NaCl solution and 0.9% NaCl solutions were also used as controls. In all cases 1 ml doses were introduced intraperitoneally into white Swiss mice (Wistar strain).

The following standard chemical tests were used: Biuret, ninhydrin, Molisch, and Benedict's. Van Gieson's picrofuchsin and Mallory's aniline blue stains were applied to nematocysts.

Polyacrylamide gels were used to obtain the electrophoresis pattern of the toxic supernatant. The procedure for preparation of the polyacrylamide gel was suggested by the Canal Instrument Corporation in their instructions for the Model 12 Electrophoresis Apparatus. The sample and stacking gels were prepared using a buffer solution of pH 6.8–7.0; the separating gel was prepared using a buffer of pH 8.8–9.0. At the anode and cathode the pH of the buffer was 8.2–8.4.

Results

Over the past seven years it has been observed that contacts of different individuals with the summer nettle produce effects varying from a very faint burning sensation, barely detectable, and erythema to a severe development of angry red wheals accompanied by systemic symptoms such as nausea, respiratory distress, *etc.* On numerous occasions live nettles were deliberately permitted to come in contact with the skin of the biceps area of the arm. In some cases this produced only the faintest stinging sensation with little or no accompanying or subsequent erythema. Many individuals, however, generally showed a somewhat more severe allergic reaction.

The present study revealed four types of nematocysts. Batteries of holotrichous isorhizas (several sizes) and larger atrichous isorhizas are distributed fairly evenly over the exumbrella, oral lobes, and tentacles. The capsules of the holotrichous isorhizas range in length from 8 to 21 μ and in width from 3 to 18 μ. The tubes of these when discharged measured from 200 to 500 μ in length, 0.5 μ in diameter. The capsules of the atrichous isorhizas range from 20 to 25 μ in length and are about 15 μ in width. Most of the tubes when discharged measured from 500 to 2000 μ in length and from 0.5 to 1.5 μ in diameter. The euryteles have capsules which range from 10 to 12 μ in length and 5 to 6 μ in width. The tubes measure 50 to 150 μ in length and approximately 0.5 μ in width. The butt is 10 to 12 μ in length and approximately 1.5 μ in width. A fourth type has a round capsule 15 to 18 μ in diameter with tube length of 150 to 250 μ and diameter of 2 μ. Additional round nematocysts were observed with a diameter of 4 to 8 μ which may be smaller forms of the fourth type.

Various methods were tried in order to effect discharge of nematocysts from both living nettles and also isolated undischarged nematocysts. Immersion in distilled water and 1 *M* sucrose were ineffective in either case. Discharge of large numbers of nematocysts from the tentacles of living nettles occurred when formalin-acetic-alcohol fixative (FAA) or faradic electric shocks were applied, but neither of these agents caused the discharge of isolated nematocysts.

Since experiments on human skin *in situ* seemed out of the question, in order to get some idea of the penetration power of nematocysts the following experiment was set up: Two per cent pure agar containing enough Grenacher's borax-carmine

to impart a red color was poured to a depth of about one-fourth inch in Petri dishes. Several slots, each about one-half inch long and one-eighth inch wide were cut out of each of these plates. A short piece of live tentacle was placed in each slot on the bottom against one side wall. A drop of FAA was applied to effect discharge of the nematocysts. The plates were then inverted under the microscope. The colorless nematocyst tubes could easily be seen in the red agar and their lengths measured from agar wall to tip of the tubes. Well over a hundred such measurements showed that the majority of nematocysts penetrated the agar from 200 to 400 μ. Most of the tubes took an almost straight course through the agar.

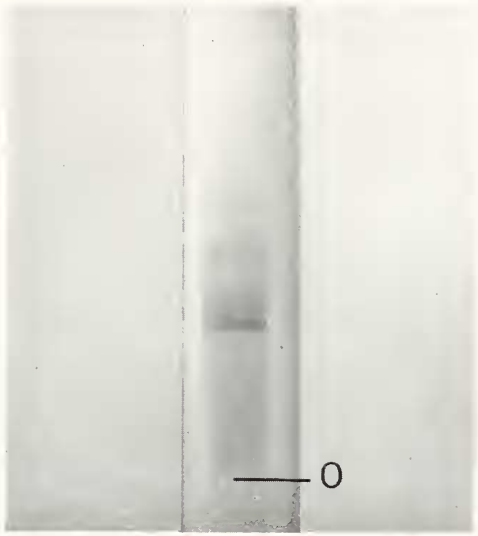

FIGURE 1. Electrophoretogram of the toxin of *Chrysaora* nematocysts obtained using polyacrylamide gels with subsequent staining with Coomassie blue stain for protein.

A large number of experiments dealing with the toxicity of nematocyst constituents to mice were performed as described under Materials and Methods. The results of these experiments show that the supernatant, after centrifugation of homogenized nematocyst suspensions at 9000 and 18,500 rpm, contained the toxin. The toxin is non-dialyzable, retaining its toxicity after salting out and subsequent dialysis. The LD 50, based on air dried residues corrected for salt content, was determined in three separate experiments to be 19 μg/g, 15 μg/g, and 16 μg/g of body weight, respectively. The ages of the doses of supernatant were 47, 85, and 193 days, respectively. It appears from a comparison of these figures that storage in a frozen state has little effect on toxicity. The residue obtained after removal of salt and after freeze-drying the supernatant was found to kill mice when administered at a level of 7.5 μg/g of body weight. Washed undischarged capsules, capsular debris, and tubes had no effect on mice; even the fine sediment after centrifugation at 18,500 rpm was non-toxic.

Various chemical tests were applied to undischarged nematocysts, capsular and tube debris, supernatant, and residue following centrifugation. The tests indicate

that: (1) thoroughly washed capsular and tube debris are negative to ninhydrin, Biuret, Molisch, and Benedict's tests before and after boiling; (2) the capsular contents (supernatant) are ninhydrin and Biuret positive but give negative results with Molisch and Benedict's reagents; (3) the residue following centrifugation at 18,500 rpm is negative to all of the tests; (4) the fluid contents of undischarged nematocysts are Biuret positive; (5) the capsules, capsular debris, and tubes do not stain appreciably, if at all, with aniline blue or picrofuchsin.

Supernatant containing toxin was thawed from the frozen state and subjected to electrophoresis using polyacrylamide gel and subsequently stained with Coomassie blue stain for protein. The resulting electrophoretogram is shown in Figure 1. One main band can be seen indicating one major protein fraction. Three faint bands may also be observed. Repetition using the supernatant from nematocysts obtained from another collection of nettles confirmed this picture.

Discussion

Burnett *et al.* (1968) observed four types of nematocysts from *Chrysaora*: Type I, oval atrichous isorhizas; Type II, round structures; Type III, euryteles; Type IV, small round structures. Halstead (1965) in a table adapted from several authors records three types of nematocysts from *Chrysaora*: atrichous isorhizas, holotrichous isorhizas, and heterotrichous microbasic euryteles. The last type is followed by a question mark indicating uncertainty. In general our observations agree with those of Burnett *et al.* (1968) except that they do not record holotrichous isorhizas. The euryteles observed by us do not appear to be heterotrichous microbasic euryteles but probably fit better in the eurytele class than any other.

Several methods for causing nematocyst discharge from living nettles have been used. Kline and Waravdekar (1960) used electric shock to effectively produce discharge of nematocysts of *Hydra littoralis*. More recently Barnes (1967) employed electrical shock with success on *C. fleckeri, C. quadrigatus* and *Cyanea capillata*. We have found that faradic shocks are quite effective over small areas of the tentacles of *Chrysaora*. FAA fixative gave good results but distilled water and 1 *M* sucrose were of little value.

Phillips and Abbott (1957) effected discharge of isolated nematocysts of *Metridium senile fimbriatum* by subjecting this anemone to various chemical agents: distilled water; methylene blue; weak acids; weak bases; sucrose; and glycerine. Burnett *et al.* (1968) compared the effectiveness of a number of physical and chemical agents in producing nematocyst rupture (not discharge) in *Chrysaora*, among them grinding; freeze thaw; distilled water; salts, acids; bases, cholinergic and adrenergic drugs; heat; and sonication. In contrast with the above results we were able to observe little or no discharge or rupture with distilled water, 1 *M* sucrose, FAA fixative, or faradic shock. Sonication of suspensions of nematocysts resulted in somewhat better than 50% rupture. The homogenization of frozen nematocyst suspensions, the procedure finally adopted, was found to be much more efficient than the method of Lane and Dodge (1958), yielding as high as 75% rupture.

Our studies of the penetration of nematocyst tubes into agar are the first to be made with *Chrysaora*. However, Cleland and Southcott (1965) record some

work on the discharge of nematocysts of Australian species of jellyfishes into human skin and the pathological effects. Barnes (1967) made observations on the penetration of nematocysts of *Chironex, Chiropsalmus* and *Cyanea* into various materials: human amnion; sheep intestine; hog stomach; fish swim bladders; synthetic sausage casings; latex; cured rubber; cellulose and various polyethylene and polyvinyl films. Further investigations in addition to those noted here should give much useful information for different species of cnidarians relative to the force of discharge of nematocysts, depth of penetration of tubes and the paths taken in test materials of various kinds.

As stated earlier in this paper a number of investigators (Lane and Dodge, 1958; Lane, 1960; Welsh, 1961; Burnett *et al.* 1968; Shapiro, 1968; Endean *et al.* 1968; Crone and Keen, 1969) have found the toxins of various cnidarians to be protein in nature. Burnett *et al.* (1968) believe (p. 335) that "the toxin factor of *Chrysaora* is a protein complex or is associated with a protein." The present work leaves little doubt that the toxin of *Chrysaora* is a protein or several proteins.

Burnett *et al.* (1968) state further that after nematocyst rupture (p. 335) "the toxin can be recovered in significant amounts in the sediments," . . . (p. 336) "that the toxin is membrane bound," . . . and (p. 335) "that the toxin is not released as a free fluid after nematocyst rupture." In contrast, the results of the present experiments show that the toxin is (1) contained in the undischarged nematocysts and is released as a free fluid after rupture which agrees with the findings of Barnes (1967) and Endean *et al.* (1968) for other species of jellyfishes; (2) the toxin is not membrane bound (that is, to the capsules); and (3) the toxin is not present in thoroughly washed empty capsules, capsular fragments, tubes, nor in sediments after centrifugation at 9000–18,500 rpm which Endean *et al.* (1968) also indicate is true of the nematocysts of *C. fleckeri.* The differences in the findings of Burnett *et al.* (1968) and the results obtained in the present work might be due to differences in procedures followed in the isolation of the toxin.

Our LD 50 determinations in general agree with those of Burnett *et al.* (1968). They conclude, however, that toxicity loss resulted from freezing refrigeration of nematocysts. The LD 50 values reported in the present work for three separate experiments indicate that storage in the frozen state for better than six months had little effect on the toxicity of nematocyst contents.

It has been suggested by several investigators, Lenhoff, Kline and Hurley (1957), Phillips (1956), Kline (1961), Goldher, Burnett, Stone and Dilaimy (1969), that the capsules of various Cnidaria are composed of, or contain, a protein of the collagenous group. If this is so, *Chrysaora* material would appear to be different from vertebrate collagen since the capsules do not stain with aniline blue nor picrofuchsin which are accepted stains for vertebrate collagen.

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Summary

1. The response of the human body to the toxin of the summer nettle, *Chrysaora quinquecirrha,* is allergic in nature.

2. Four types of nematocysts were identified: atrichous isorhizas; holotrichous isorhizas; euryteles; and a round type.

3. Application of faradic electrical shocks and the fixative FAA were effective in producing discharge of nematocysts in living nettles but not of isolated nematocysts.

4. The tubes of freely discharged nematocysts measured from 500–2000 μ in length. When discharged into 2% agar, the majority of tubes penetrated a distance of only 200–400 μ.

5. An efficient method of isolation and rupture of the nematocysts with subsequent isolation of the toxin is described.

6. The toxin is non-dialyzable. It gives positive ninhydrin and Biuret tests but negative Molisch and Benedict's tests.

7. The toxin is a protein or several proteins and is contained in the free fluid discharged from the nematocyst capsules. It is not membrane bound.

8. When inoculated intraperitoneally into white mice, the toxin gave an LD 50 of 19 μg/g, 15 μg/g, and 16 μg/g in three experiments.

9. There is little if any loss in toxic activity of nematocyst contents after storage in the frozen state over a six-month period.

10. The toxicity of the supernatant is retained after freeze-drying.

11. Thoroughly washed empty capsules, capsular fragments, and tubes do not appear to have any toxic effect when injected intraperitoneally into white mice.

12. Thoroughly washed empty nematocyst capsules, capsular fragments and tubes, and residues from high speed centrifugation gave negative results when tested with ninhydrin, Biuret, Molisch, and Benedict's reagents.

13. Empty washed capsules, capsular debris, and tubes do not stain with aniline blue nor picrofuchsin which are accepted stains for vertebrate collagen.

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